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Partial Amplification of the Measles Virus Nucleocapsid Gene From Stored Sera and Cerebrospinal Fluids for Molecular Epidemiological Studies

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The analysis of stored sera for retrospective molecular epidemiological studies provides a powerful tool to investigate strain variation in measles viruses that had circulated up to 20 years ago. For this purpose, a rapid and simple method for extraction of RNA from stored sera and cerebrospinal fluids (CSF) was developed. When used on sera and CSFs that have been frozen for as long as 20 years, this method proved to be more efficient than established techniques. The extracted RNA was reverse transcribed into cDNA by using random hexamer primers. The PCR amplification of the 3′ terminus of the nucleocapsid gene (N) was divided into two overlapping fragments of 375 and 384 bp length, covering the entire region of interest. This region is thought to have the highest variability within the MV genome and has previously been shown to be suitable for strain characterization. The resulting PCR fragments were sequenced manually by using standard methods without the need of further clean-up steps. ***J. Med. Virol. 56:174–177, 1998.***

KEY WORDS: measles virus; serum-RT-PCR; sequence analysis

INTRODUCTION

Only one serotype of measles virus has been described to date. However, on the basis of gene sequences, wild-type (wt) measles viruses have been divided into several distinct genetic groups [Rima et al., 1995; Rota et al., 1994,1996], the majority of which seem to be restricted to certain geographical areas [Rota et al., 1994; Rima et al., 1995]. However, the increase in worldwide travel may contribute to the importation of geographically restricted virus groups into countries that have previously succeeded in interrupting transmission of indigenous measles viruses within their borders [Rota et al., 1996; Bellini and Rota, 1998]. Molecular epidemiological studies are gaining in importance as more countries are beginning to implement measles virus control and elimination programmes. Only by the ongoing genotyping of circulating viruses will it become possible to distinguish imported versus indigenous virus strains, a crucial step in monitoring the success of vaccination campaigns and measles virus elimination efforts [Rota et al., 1996; Bellini and Rota, 1998]. The analysis of stored sera can therefore provide valuable molecular epidemiological data on measles virus strains that were circulating at a time before mass vaccination programmes successfully reduced the incidence of wild-type (wt) measles virus in certain regions.

The standard method for determining sequence variation between measles virus strains for molecular epidemiological purposes has been sequence analysis of the 3′ terminus of the N gene and the haemagglutinin (H) gene [Outlaw and Pringle, 1995; Rima et al, 1997]. For amplification of the fragments, RT-PCR is performed routinely on RNA extracted from either virus-infected tissue culture or directly from clinical samples such as urine, throat swabs, nasopharyngeal secretions, serum, and CSFs [Outlaw and Pringle, 1995; Rota et al., 1995].

A rapid method is described for extraction of RNA and subsequent amplification of the 3′-terminus of the N gene from sera and CSFs that have been stored for up to 20 years. RNA was reverse transcribed into cDNA using random primers and cDNA was then amplified with measles virus specific primers that allowed for amplification of two overlapping fragments, covering the entire region of interest.

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TABLE I. Nucleotide Sequence of Oligonucleotide Primer Pairs and Their Position in the Nucleocapsid Gene

Primers	Orientation	Position	Sequence
N16[a]	forward	987–1017	5′ TTCAGAACAAGTTCAGTGCAGGATCATACC 3′
N17	reverse	1363–1334	5′ CCTCGACTCTGTTTGACCCTCCTATCTTCC 3′
N18	forward	1235–1264	5′ TGAGGACAAGATCAGTAGAGCCGGTTGGACC 3′
N8	reverse	1619–1599	5′ GGCCTCTCGCACCTAGTCTAG 3′

[a]All PCR primers were also used for sequencing.

MATERIALS AND METHODS

Clinical Samples

Recent sera and CSFs form Durban, Port Elizabeth, and Windhoek (Namibia) were confirmed to be IgM positive, or in the case of PE1/97 IgG positive, by the Enzygnost Anti-Measles Virus IgG/IgM ELISA (Behring) (see Table II). Specimens from 1990 and earlier had unknown IgM status but were generally collected during clinically confirmed measles outbreaks within South Africa. All sera and CSFs had been stored at –20°C for various periods of time and had probably undergone several freeze-thaw cycles. Sera that were negative for measles virus IgM antibodies served as controls for RNA extraction and subsequent RT-PCR.

Extraction of RNA

Serum or CSF (200 μl) was thawed on ice and then added to 600 μl of chilled Guanidinium thiocyanate solution (4 M guanidinium thiocyanate dissolved in nuclease-free water, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) lauroyl sarcosine and 0.1 M 2-mercaptoethanol). After incubation on ice for 5 min, a 1/10 volume of 3 M sodium acetate, pH 5.0 was added and RNA then extracted with 500 μl of water-saturated phenol and 100 μl of chloroform. Nucleic acid was precipitated for a minimum of 1 hr in the presence of isopropanol at –20°C. Centrifugation at 20,000 × g for 10 min resulted in a pellet that was washed three times with 70% ethanol. The extracted nucleic acids were dried at 56°C for 3 min and at 37°C for 5 min. RNA was resuspended in 12 μl of nuclease-free water and used directly for RT-PCR.

RT-PCR and Primers

To 12 μl of extracted nucleic acid, 0.2 μg of random hexamer (N_6) primers (Boehringer Mannheim, Mannheim, Germany) were added, the sample incubated at 70°C for 10 min, and then chilled on ice for 5 min. To the annealed primer-template mix, 4 μl of 5× RT buffer containing 250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, and 5 mM DTT, pH 8.5 (Boehringer Mannheim), 0.25 μl of each 10 μM deoxynucleoside triphosphate (dNTP) and an additional 2 μl of 0.1M DTT were added. After incubation for 10 min at 25°C and for 2 min at 42°C, reverse transcription was initiated by the addition of 20 U of AMV-RT. Incubation was continued for 50 min followed by the inactivation of the AMV-RT for 5 min at 85°C. 3–5 μl of cDNA were added to a PCR master mix containing 10 μl of 10× Taq buffer (100 mM of Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3, Boehringer Mannheim), an additional 3 μl of 25 mM $MgCl_2$, 10 μM of each dNTP, 60 pmol of each forward and reverse primer, and 5 U of Taq polymerase (Boehringer Mannheim) in a total volume of 100 μl. Measles virus specific primers (Table I) amplified two overlapping fragments of the 3′ terminus of the N gene of 375 bp and 384 bp in length, respectively. PCR cycling conditions were as follows: 95°C for 1 min followed by 30 cycles of 94°C for 50 sec, 50°C for 1 min, and 72°C for 1min with an additional 1 sec per cycle and a final incubation for 5 min at 72°C.10 μl of PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. Where first-round PCR products were poorly visible, a second round PCR was performed using the above mentioned primers and conditions, with 5–10 μl of the first-round product used as template.

Sequence Analysis of PCR Products and Alignment of Amino Acids

PCR products (5 μl) were sequenced directly using the Sequenase PCR product sequencing kit (Amersham, USB, Buckinghamshire, UK) according to the manufacturer's instructions. Predicted amino acid changes were aligned using the DNASIS for Windows (WDNASIS, version 2.5, Hitachi).

RESULTS AND DISCUSSION

Fourteen sera and 3 CSFs collected in 1978 during clinically confirmed measles outbreaks in South Africa were selected for extraction of RNA and subsequent amplification of the 3′ terminus of the measles virus N gene. Of the 17 specimens analysed, 4 (3 sera and 1 CSF) resulted in PCR products that were suitable for direct sequencing analysis. These serum and CSF samples had been stored for 20 years at –20°C and were likely to have been frozen and thawed several times due to use of the samples for other studies. Considering the substantial degradation of RNA that occurs in samples that have been stored for prolonged periods of time and that have been frozen and thawed numerous times, positive amplification in 24% of cases was satisfying. However, the extent of RNA degradation did not permit the amplification of fragments longer than 350–400 bp in most of these samples (data not shown).

Out of a total of eight specimens collected in 1997 in Windhoek, Durban, and Port Elizabeth, measles virus specific DNA could be amplified from six (75%). These specimens were included in the study for molecular

TABLE II. Clinical Samples Used in This Study

Name[a]	Source	IgM status	Origin[a]	Genotype
SA 168/78	serum	?	Soweto, SA	D5
SA 280/78	CSF	?	Soweto, SA	I
SA 289/78	serum	?	?, SA	I
SA 361/78	serum	?	SA	I
Dbn 1/97	serum	+	Durban, SA	I
Wdk 28/97	CSF	+	Windhoek, Namibia	I
Wdk 91/97	serum	+	Windhoek, Namibia	I
PE 1/97[b]	CSF	IgG+	Port Elizabeth, SA	I
Jhb 1/90	serum	+	Johannesburg, SA	I

[a]SA = South Africa; Dbn = Durban, South Africa; Wdk = Windhoek, Namibia; PE = Port Elizabeth, South Africa; Jhb = Johannesburg, South Africa.
[b]Analysis of a serum sample taken from the same patient (PE 1/97) revealed identical sequences compared to the CSF specimen.

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SCH          1 KVSSTLASEL GITAEDARLV SEIAMHTTED KISRAVGPRQ AQVSFLHGDQ        50
JHB2-88      1 .......... .......... .......... .......... ..........        50
JHB1-89      1 .......... .......... .......... R......... ..........        50
JHB14-95     1 .......... .......... .......... R......... .....I....        50
SA168-78     1 .......... .......... .......... R......... ..........        50
SA280-78     1 .......... .......... .......... R......... .....I....        50
SA289-78     1 .......... .......... .......... R......... .....I....        50
SA361-78     1 .......... .......... .......... .......... .....I....        50
DBN1-97      1 .......... .......... ..T....... R......... .....I....        50
WDK28-97     1 .......... .......... .......... .......... ..........        50
WDK91-97     1 .......... .......... .......... R......... ..........        50
PE1-97       1 .......... .......... .......... R......... ..........        50
JHB1-90      1 .......... .......... .......... R......... .....I....        50

SCH         51 SENELPRLGG KEDRRVKQSR GEARESYRET GPSRASDARA AHLPTGTPLD       100
JHB2-88     51 .......... .......... .......... .......... ..........       100
JHB1-89     51 ......G... .......... .......... .S..T..... .....S....       100
JHB14-95    51 ......G... .......... .........A .S........ .....S....       100
SA168-78    51 .......... .......... .......... .S..T..... .....S....       100
SA280-78    51 ......G... .......... .........A VS........ .....S....       100
SA289-78    51 ......G... .......... .........A .S........ .....S....       100
SA361-78    51 ......G... .......... .........A .S........ .....S....       100
DBN1-97     51 ......G... .......... .........A .S........ .....S....       100
WDK28-97    51 ......G... .......... .......... .S........ .....S....       100
WDK91-97    51 ......G... .......... .........A .S........ .....S....       100
PE1-97      51 ......G... .......... .......... .S........ ..P..SM...       100
JHB1-90     51 ......G... .......... .........A .S...N.... .....S....       100

SCH        101 IDTASESSQD PQDSRRSADA LLRLQAMAGI TEEQGSDTDT PIVYNDRNLL       150
JHB2-88    101 ....T..... .......... .......... S......... ..........       150
JHB1-89    101 .......... .......... .......... S......... .R.....D..       150
JHB14-95   101 .......G.. .......... .......... L........I .R.....D..       150
SA168-78   101 .......... .......... .......... S......... .R.....D..       150
SA280-78   101 .......G.. .......... .......... L........I .R.....D..       150
SA289-78   101 .......... .......... .......... L........I .R.....D..       150
SA361-78   101 .......G.. .......... ........V. L........I .R.....D..       150
DBN1-97    101 .......... .......... .......... L........I .R.....D..       150
WDK28-97   101 .......... .......... ......K... L......... .R.....D..       150
WDK91-97   101 .......... .......... .......... L......... .R.....D..       150
PE1-97     101 .......G.. .......... .......... L......... .R.....D..       150
JHB1-90    101 .......G.. .......... .......... L......... .R.....D..       150
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Fig. 1. Alignment of predicted amino acid changes within the carboxyl-terminal 150 amino acids of the measles virus N protein. Strains are as described in Table II or as published previously (Kreis et al., 1997). A dot indicates the same residue as in the vaccine strain Schwarz (Sch).

epidemiological analysis of MV strains collected in regions other than Johannesburg. As clinical samples suitable for virus isolation were not available from these locations, it was useful to obtain sequence data from RNA extracted from sera or CSFs.

Figure 1 shows the amino acid (aa) alignment of all samples analysed in this study as well as three South African isolates (Jhb2/88, Jhb1/89, Jhb14/95) representing the three different genetic groups currently circulating in South Africa that have been described before (Kreis et al., 1997). None of the viruses analysed here shared similarities with the vaccine-like virus

Jhb2/88 or any other vaccine or vaccine-like strains. Jhb1-89 is a representative of a small group of six South African viruses (group D5) that seem to have been more common in the 1970s and 1980s (Kreis et al., 1997). SA168-78 shared 8 out of 9 amino acid (aa) changes with Jhb1/89 indicating a close relationship. Phylogenetic analysis confirmed the similarity of SA168/78 with this group of viruses (data not shown). PE1/97 and Wdk28/97 carry some unique aa changes that are not shared by any other viruses described in Figure 1. This finding was confirmed by phylogenetic analysis of the sequences revealing that these two viruses were indeed different and did not group within any of the South African genetic lineages described before (data not shown).The majority of viruses listed proved to be closely related to the predominant group of viruses (group I) that includes ~90% of isolates currently circulating in South Africa (Kreis et al., 1997).

Where sufficient quantities of serum (4/14) were available, the RNA extraction method described by Chomczynski and Sacchi [1987] as well as the RT-PCR conditions described previously [Rota et al., 1994] were carried out in parallel with the method described above. Neither of these standard approaches yielded PCR products suitable for sequencing analysis (data not shown).

Worldwide, efforts are being made to reduce the incidence of measles virus infections. Some countries in the developed world are moving toward the elimination of measles virus from within their borders, or have succeeded in interrupting transmission of indigenous virus, as has been shown for the United States [Rota et al., 1996; Bellini et al., 1998]. Nevertheless, most developing countries are only now beginning to embark on MV control programmes such as mass vaccination campaigns and strategies to improve routine vaccine coverage. For countries that currently do not undertake molecular epidemiological surveillance of measles virus, the amplification of the 3′-terminus of the N gene using the PCR described here could be a useful tool in obtaining retrospective epidemiological data on stored sera. This would allow for sequence analysis of the amplified fragments in order to establish the characteristics of measles virus strains that have been circulating in that country over time. The knowledge of the genetic characteristics of measles virus strains prior to implementation of control measures and mass vaccination campaigns is essential for distinguishing imported from indigenous virus strains.

Most techniques reported to date that allow for partial amplification of the measles virus genome from clinical samples such as sera and CSFs were used only on fresh samples that had never been frozen, or proved to be rather time-consuming [Matsuzono et al., 1994]. In addition, other methods amplified only conserved regions [Jin et al., 1996] and resulted in PCR products of <200 bp in length [Nakayama et al., 1995], or required further DNA purification prior to sequence analysis [Nakayama et al., 1995; Jin et al., 1996].

In summary, the method for RNA extraction and RT-PCR of the 3′ terminal 450 bp of the N gene described here is rapid, relatively simple, and yields PCR products that could be used directly for sequencing analysis. Apart from the retrospective analysis of measles virus using stored material, this technique can further be used to obtain sequence data from recent sera in situations where specimens for virus isolation are not available.

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